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Nocistatin inhibits pregnant rat uterine contractions *in vitro*: Roles of calcitonin gene-related peptide and calcium-dependent potassium channel

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ABSTRACT

The endogenous neuropeptide nociceptin/orphanin FQ, translated from the prepronociceptin gene, exerts a contraction-inhibitory effect on the rat uterus. As nocistatin has been reported to cause functional antagonism of the pro-nociceptive effects of nociceptin, we set out to investigate its effects on the pregnant rat uterus and to elucidate its signalling pathway. The expression of prepronociceptin mRNA in the uterus and nocistatin levels in the uterus and the plasma were confirmed by RT-PCR and radioimmunoassay. The uterine levels of prepronociceptin mRNA and nocistatin were significantly increased by the last day of pregnancy, while the plasma nocistatin levels remained unchanged. In the isolated organ bath studies nocistatin inhibited the prostaglandin- and the KCl-evoked contractions in the uterus dose-dependently. This latter effect was decreased by preincubation with capsaicin. Incubation with calcitonin gene-related peptide after capsaicin treatment caused an elevation in the contraction-inhibitory effect of nocistatin. The effect of nocistatin was also decreased by the Ca^{2+}-dependent K^+ channel inhibitor paxilline, against spontaneous uterine contractions. Nociceptin potentiated the action of nocistatin. Naloxone decreased the effect of nocistatin administered either alone or in combination with nociceptin. In Ca^{2+}-poor environment, this effect of naloxone was suspended. Enzyme immunoassay for the uterine intracellular cAMP levels partially confirmed the results of *in vitro* contractility studies. We conclude that nocistatin, generated locally in the uterus, exerts an inhibitory effect, the mechanism being mediated in part by Ca^{2+}-dependent K^+ channels, the elevation of cAMP levels and sensory neuropeptides.

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1. Introduction

After the identification of the endogenous ligand nociceptin (N/OFQ) of the nociceptin receptor (NOP receptor), another neuropeptide originating from the same precursor protein prepronociceptin (PNOC) was isolated (Okuda-Ashitaka et al., 1998). It was named nocistatin, with reference to its first effects described in the central nervous system, as a functional antagonist of N/OFQ (Zeilhofer et al., 2000). It has been demonstrated that nocistatin binds to a binding site that is distinct from the NOP receptor (Okuda-Ashitaka and Ito, 2000; Johnson and Connor, 2007). Nocistatin inhibits 5-hydroxytryptamine release via a $G_{i/o}$ protein-mediated pathway (Johnson and Connor, 2007; Fantin et al., 2007).

The mRNA for *PNOC* is widely expressed in the brain (Neal et al., 1999; Martin et al., 1998) and in the spinal cord (Mollereau et al., 1996). Nocistatin attenuates the allodynia and hyperalgesia caused by the intrathecal administration of N/OFQ and PGE_2 (Minami et al., 1994a, 1994b; Taiwo and Levine, 1988; Okuda-Ashitaka et al., 1998). Both N/OFQ and nocistatin have impacts on memory and learning (Hiramatsu and Inoue, 1999). Nocistatin suppresses appetite (Olszewski et al., 2000) and induces gastric mucosal protection (Zádori et al., 2008).

Endogenous opioid peptides are involved in the induction of the GnRH surge (Foradori et al., 2007). N/OFQ is present in several pathological conditions, such as the female fibromyalgia syndrome (Anderberg et al., 1998) and postpartum depression (Gu et al., 2003), and it is a paracrine mediator of the FSH effects in the regulation of spermatogenesis (Eto et al., 2012). A high expression of N/OFQ in adenomyosis may be one of the casual factors of dysmenorrhoea (Hou et al., 2011). N/OFQ regulates the LH surge and ovarian function (Sinchak et al., 2006). The N/OFQ–NOP receptor system modulates and coordinates reproductive behaviour and physiology through actions in the limbic system and hypothalamus (Sinchak et al., 2006).

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A number of endogenous peptides participate in the regulation of uterine contractility, including oxytocin and tachykinin promoting contractions (Popescu et al., 1985; Pennefather et al., 2004; Moodley et al., 1999; Collins et al., 2002). Other neuropeptides, such as calcitonin gene-related peptide (CGRP), contribute to relaxation of the uterus smooth muscle (Shew et al., 1991; Pennefather et al., 1990; Klukovits et al., 2004). CGRP and tachykinin are localised in the capsaicin-sensitive sensory nerve endings. As labour and delivery are extremely painful, we decided to evaluate the effects of PNOC-derived peptides which have a role in pain modulation, in order to establish whether they additionally influence uterine contractility. We earlier reported that the endogenous peptide N/OFQ inhibits uterine contraction in the pregnant rat (Klukovits et al., 2010).

Our present aims were to detect the expression of PNOC mRNA and nocistatin in the late-pregnant rat uterus and to clarify the effects of nocistatin alone or in combination with N/OFQ *in vitro*. We hypothesised that Ca^{2+}-activated K^+ channels ($K_{Ca}1.1$) and outward K^+ currents, the changes in intracellular cAMP levels, and sensory neuropeptide CGRP may be involved in the uterine effects of nocistatin. Additionally, we aimed to investigate the opiate-receptor-independent peripheral actions of nocistatin.

2. Materials and methods

2.1. Animals

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV./01758-2/2008) and under the control of the ISO-9001:2008 Quality Management System.

Sexually mature female Sprague-Dawley rats (body mass: 160–200 g, 50–60 days old) were mated in the early morning hours. Copulation was confirmed by the presence of a copulation plug or spermatozoa in the vagina. The day of copulation was considered to be the first day of pregnancy. The animals were housed in temperature (20–23 °C), humidity (40–60%) and light (12 h of light, 12 h of dark)-regulated rooms, with water and food intake available *ad libitum*.

2.2. Real-time reverse transcription polymerase chain reaction (RT-PCR) studies

On selected days of late pregnancy (days 18, 20 and 22), rats were killed by CO_2 inhalation, the uteri were excised and trimmed of fat, the feto-placental units were removed and the endometrium was denuded. The tissue samples were frozen immediately in liquid nitrogen, and then stored at −80 °C until analysis. The frozen samples were ground with a Micro-Dismembrator S homogeniser (Sartorius, Germany), and the total RNA was isolated with the TRIsure Kit according to the manufacturer's instructions. RNA purity was controlled via the optical density at 260/280 nm with a BioSpec Nano instruments (Shimadzu, Japan); all samples exhibited an absorbance ratio in the range 1.6–2.0. RNA quality and integrity were assessed by agarose gel electrophoresis. One microgram of each sample of total RNA was used for reverse transcription and amplification (TaqMan RNA-to-C_T 1-Step Kit and the Sensi FAST Probe Hi-Rox One-Step Kit). The following primers were used: assay ID Rn01637101_m1 for *PNOC*, Rn 00667869_m1 for *ß-actin* and Rn 01775763_g1 for *GAPDH* as endogenous controls. RT-PCR was performed by using the ABI StepOne Real-Time cycler. The fluorescence intensities of the probes were plotted against PCR cycle numbers. The amplification cycle displaying the first significant fluorescence signal increase was defined as the threshold cycle (C_T).

There is an ongoing debate about the applicability of beta-actin as a gene reference in the pregnant rat uterus, with no reassuring conclusion as yet (Kelly et al., 2003). Thus we have repeated our PCR studies also with GAPDH as a gene reference. We did not find differences in the change of *PNOC* mRNA when GAPDH was used as compared with beta-actin.

2.3. Radioimmunoassay (RIA) for nocistatin in the rat uterus and plasma

In RIA studies, blood and uterine samples from non-pregnant and 15-, 18-, 20- and 22-day pregnant rats were used.

Blood was collected in K_3-EDTA containing vacutainers (OMKER, Hungary) and Aprotinin (0.6 TIU/ml) was added immediately as a protease inhibitor. Plasma was separated by centrifugation at 3000 rpm for 10 min and samples were kept frozen at −80 °C until direct analysis by radioimmunoassay (RIA).

1000 µl aliquots of plasma samples were mixed with equal volume of 1% v/v trifluoroacetic acid (TFA I.), centrifuged at 1600*g* for 20 min at 4 °C. The acidified samples were loaded onto C18 Sep-Pack cartridges (ABL&E JASCO Hungary Ltd.), washed twice with TFA I., and then eluted with 60% acetonitrile in 0.1% TFA. Samples were freeze dried by centrifugation (SAVANT, Instruments, Inc., Farmingdale, NY, USA). The reconstituted eluate was subjected to RIA using commercially available ^{125}I-Nocistatin RIA kit with minimum sensitivity of 10 pg/ml. Data were evaluated by RIA-Mat 280 (Byk-Sangtec, Dietzenbach, Germany).

Extraction of nocistatin was carried out by a validated method adapted from Eun-Mee et al. (1999) and Hofbauer et al. (2000). Uterus samples were treated with 1 M acetic acid (250 mg/ml), placed in a 95 °C water bath for 5 min then cooled in ice-cold water bath for 10 min and homogenised by an Ultra Turrax T25 Janke&Kunkel homogeniser (IKA Labortechnik, Staufen, Germany) at 20,000 rpm for 10 s, followed by an ultrasound homogenisation (Labsonic 2000, B.Braun, AG, Melsungen Germany) for 10 s. Samples were replaced in the 95 °C water bath for 5 min then cooled in ice-cold water bath, and centrifuged at 4 °C for 10 min at 12,000*g* in an Eppendorf centrifuge (A. Hettich, Tuttlingen, Germany). Aliquots of 1.0 ml from the supernatants were treated as described for plasma samples.

During the validation of the extraction procedure stability of nocistatin, reproducibility, recovery and linearity of recovery in the 10–500 pg/100 mg tissue concentration range were determined. Recovery was 89 ± 0.87%, and all the other parameters were in the normal range.

2.4. In vitro contractility studies

2.4.1. Uterus preparation

On day 22 of pregnancy (at term), the rats were killed by CO_2 inhalation, and the uteri were removed and prepared for the *in vitro* contractility assay as reported previously (Klukovits et al., 2010). Briefly, the isolated uterine horns were immediately placed in an organ bath (de Jongh solution; containing in mM: 137 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 4 Na_2HPO_4, 6 glucose; pH 7.4) perfused with a mixture of 95% oxygen and 5% carbon dioxide (carbogen) and trimmed of fat, and the feto-placental units were removed. The temperature was maintained at 37 °C. Four rings 0.5 cm long were sliced from the middle part of each horn, including implantation sites, and tested in parallel; they were mounted vertically in the above-mentioned organ bath containing 10 ml of de Jongh solution. After mounting, the initial tension was

set at 1.5 g and the rings were equilibrated for 60 min, with a solution change every 15 min.

2.4.2. Nocistatin studies

In the isolated uterine rings, rhythmic contractions were elicited with 25 mM KCl or with 10 nM oxytocin or with 1 μM $PGF_{2\alpha}$. Without washing out the contractile agents, the effects of nocistatin on the uterine contractions were tested in the concentration range 10^{-12}–10^{-6} M, in a noncumulative manner (as regards its peptide characteristic). After each concentration of nocistatin, the rings were washed 3 times, allowed to recover for 5 min, and then contracted again with the above-mentioned agents. Following the oxytocin- and $PGF_{2\alpha}$-induced contractions, the contraction-inhibitory effect of nocistatin was also investigated in the presence of N/OFQ (10^{-8} M).

Following the KCl-induced contractions, the contraction-inhibitory effect of nocistatin was investigated in the presence of N/OFQ (10^{-8} M) and/or naloxone (10^{-8} M). The most potent inhibitory effect of nocistatin was found in the KCl-induced contractions; this agent was therefore used to investigate the possible mechanism of nocistatin in further studies. As naloxone induces an increase in inward Ca^{2+} currents (Kai et al., 2002), we conducted a series of experiments in a low Ca^{2+} environment in order to investigate whether the inhibitory effect of naloxone on the nocistatin-induced uterus relaxation is mediated by the opening of inward rectifying Ca^{2+} channels. Thus the joint effect of nocistatin and naloxone was studied in a modified de Jongh buffer, containing half the Ca^{2+} concentration (0.5 mM $CaCl_2$) of the standard de Jongh buffer (Hajagos-Tóth et al., 2009). In order to investigate the participation of the outward rectifying K^+ channels in mediating the effects of nocistatin, tests were performed in the presence of the $K_{Ca}1.1$ channel-selective blocker paxilline (5×10^{-6} M), against spontaneous uterine contractions.

The possible involvement of the sensory neuropeptide CGRP in the actions of nocistatin was also tested on uterine tissue. In this set of experiments, capsaicin (1 μM dissolved in physiological saline containing 6% Tween 80 and 8% ethanol; for 10 min) was used to deplete CGRP from the uterine sensory nerve endings (Holzer, 1991). After thorough washing out, the tissues were incubated with CGRP (0.1 μM; 20 min) (Sams-Nielsen et al., 2001) and washed again, and the effects of nocistatin were tested as above. These experiments were performed in a de Jongh solution supplemented with protease inhibitors such as phenylmethanesulfonyl fluoride (1 μM), captopril (0.1 mM), dithiothreitol (0.5 mM), soy bean trypsin inhibitor (1 mM) and aprotinin (36,000 kIU/l).

The tension of the myometrial rings was measured with a strain gauge transducer (SG-02, Experimetria Ltd., Budapest, Hungary), and recorded and analysed with the SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Budapest, Hungary). The areas under the curves of 4-min periods were evaluated; the effects of nocistatin, N/OFQ and naloxone were expressed as percentages of KCl/oxytocin/$PGF_{2\alpha}$-induced or spontaneous contractions. The dose–response curves were fitted and the geometrical mean of log EC_{50} values and maximum contraction-inhibitory values were calculated with the Prism 4.0 computer programme (GraphPad Inc., San Diego, CA, USA).

2.5. Measurement of uterine cAMP accumulation

As cAMP elevation plays a major role in the relaxation of uterine smooth muscle, we set out to investigate its possible involvement in the nocistatin signalling. Uterine tissue samples were incubated in de Jongh solution at 37 °C, perfused with carbogen. cAMP accumulation was determined in the presence of the nonspecific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 10^{-3} M; 10 min), nocistatin (10^{-8} M; 10 min) alone or in combination with N/OFQ (10^{-8} M) or naloxone (10^{-8} M), and the adenylyl cyclase activator forskolin (10^{-5} M; 10 min). The samples were then immediately frozen in liquid nitrogen and stored until the extraction of cAMP. Frozen tissue samples were ground, weighed, homogenised in 10 volumes of ice-cold 5% trichloroacetic acid and centrifuged at 1000*g* for 10 min. The supernatants were extracted with 3 volumes of water-saturated diethyl ether. After drying, the extracts were stored at -80 °C until the cAMP assay. Uterine cAMP accumulation was measured with a commercial competitive cAMP enzyme immunoassay (EIA) kit and tissue cAMP levels were expressed in pmol/mg tissue. All samples ($n=4$ in each group) were measured in duplicate in the EIA.

2.6. Materials

N/OFQ and nocistatin were purchased from PolyPeptide Laboratories France SAS, Strasbourg, France. $PGF_{2\alpha}$, naloxone, forskolin, IBMX, cAMP Enzyme Immunoassay Kit, paxilline, capsaicin, soy bean trypsin inhibitor, dithiothreitol, phenylmethanesulfonyl fluoride and captopril were from Sigma-Aldrich Ltd., Budapest, Hungary. The TRIsure Kit and the Sensi FAST Probe Hi-Rox One-Step Kit were from Bioline Ltd., Budapest, Hungary. TaqMan RNA-to-C_T 1-Step Kit, β-actin and GAPDH primers were obtained from Life Technologies, Budapest, Hungary. The ^{125}I-Nocistatin RIA kit was from Phoenix Pharmaceuticals, Inc., purchased by Izinta Ltd., Budapest, Hungary. Aprotinin (Gordox®) and oxytocin were purchased from Richter Gedeon Ltd., Budapest, Hungary.

2.7. Statistical analysis

Statistical analyses were carried out by ANOVA Newman–Keuls multiple comparison test with the Prism 4.0 computer programme (GraphPad Inc., San Diego, CA, USA). This test makes pairwise comparisons of group means. The alpha level of Newman–Keuls test is 0.05.

3. Results

3.1. Measurement of PNOC mRNA in the uterus

The myometrial *PNOC* mRNA levels increased significantly as term was approached. The PCR study showed that the levels of *PNOC* mRNA/*β-actin* mRNA and *PNOC* mRNA/*GAPDH* mRNA were the lowest on pregnancy day 18. The relative expression of *PNOC*

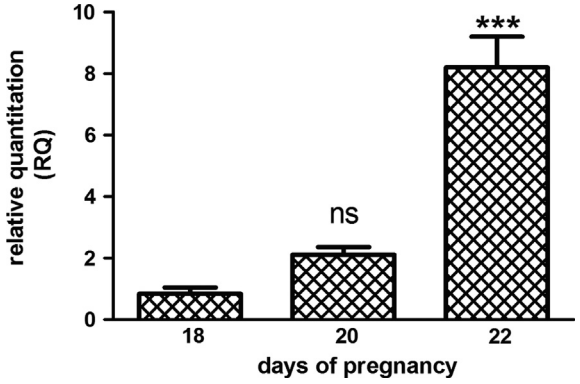

Fig. 1. Levels of expression of *PNOC* mRNA in the rat uterus on days 18, 20 and 22 of pregnancy. As term was approached, the *PNOC* mRNA level increased; there was a significant increase in the level of *PNOC* mRNA in uterus samples obtained from rats on day 22 (*i.e.* at term) as compared with that on day 20 of pregnancy ($n=3$); ***$P<0.001$, ns: non-significant. Significances are expressed relative to the previous column. The endogenous control is *β-actin*.

mRNA on day 20 was not different from that on day 18, but it was increased significantly by day 22, the day of delivery; $P<0.001$ (Fig. 1, the endogenous control was *β-actin*; we observed the same results with *GAPDH*, data not shown).

3.2. Measurement of nocistatin in the uterus and the plasma

The myometrial nocistatin levels increased significantly as term was approached. The RIA experiments revealed that the levels of nocistatin were relatively low on pregnancy days 15, 18 and 20 ($P>0.05$), then elevated significantly by day 22, the day of delivery; $P<0.05$ as compared with day 20 (Table 1). The nocistatin levels on day 22 did not differ from the non-pregnant samples. The plasma levels of nocistatin did not change as term was approached (days 15, 18, 20 and 22; $P>0.05$ in all comparisons).

Table 1
Tissue and plasma nocistatin levels in non-pregnant, 15, 18, 20 and 22 day pregnant rats ($n=6$).

Tissue	pg/100 mg uterine tissue ± S.E.M		pg/ml plasma ± S.E.M	
Non-pregnant	47.07 ± 4.66		12.07 ± 1.53	
15 day pregnant	17.49 ± 4.41	[a]	14.11 ± 1.63	ns
18 day pregnant	17.15 ± 3.03	ns	11.52 ± 1.62	ns
20 day pregnant	13.95 ± 1.82	ns	12.19 ± 1.57	ns
22 day pregnant	42.11 ± 6.27	[a]	17.59 ± 2.12	ns

[a] $P<0.05$, ns: nonsignificant. Significances are expressed relative to the value of the previous tested day.

3.3. In vitro contractility studies

3.3.1. Investigation of the contraction-inhibitory effects of nocistatin

The contraction-inhibitory effect of nocistatin was investigated in three different agonist-induced contractions (Fig. 2). The oxytocin-, $PGF_{2\alpha}$- or KCl-stimulated contractions did not decrease significantly through the experiment. Oxytocin was able to contract the uterine smooth muscle, but in the presence of oxytocin, nocistatin alone or in the presence of N/OFQ (10^{-8} M) did not significantly reduce the contractions (data not shown). In the case of $PGF_{2\alpha}$-induced contractions, nocistatin and N/OFQ displayed slight inhibitory effects (Fig. 3). There was no significant difference between the log EC_{50} values. Nocistatin alone decreased the KCl-induced contractions concentration-dependently. Co-administration of N/OFQ (10^{-8} M) with nocistatin, however, significantly increased the maximum contraction-inhibitory effect of nocistatin; $P<0.05$ (Fig. 4A). There was no significant difference between the log EC_{50} values (Table 2). The most potent inhibitory effect of nocistatin was found in the KCl-induced contractions; this agent was therefore used to investigate the possible mechanism of nocistatin in further studies.

3.3.2. Investigation of the contraction-inhibitory effects of nocistatin and naloxone in a standard and a Ca^{2+}-poor environment

In standard de Jongh solution, the maximum inhibitory effect of nocistatin was decreased by naloxone (10^{-8} M); $P<0.001$. There was no significant difference between the log EC_{50} values (Table 3). Naloxone also decreased the maximum contraction-inhibitory effect

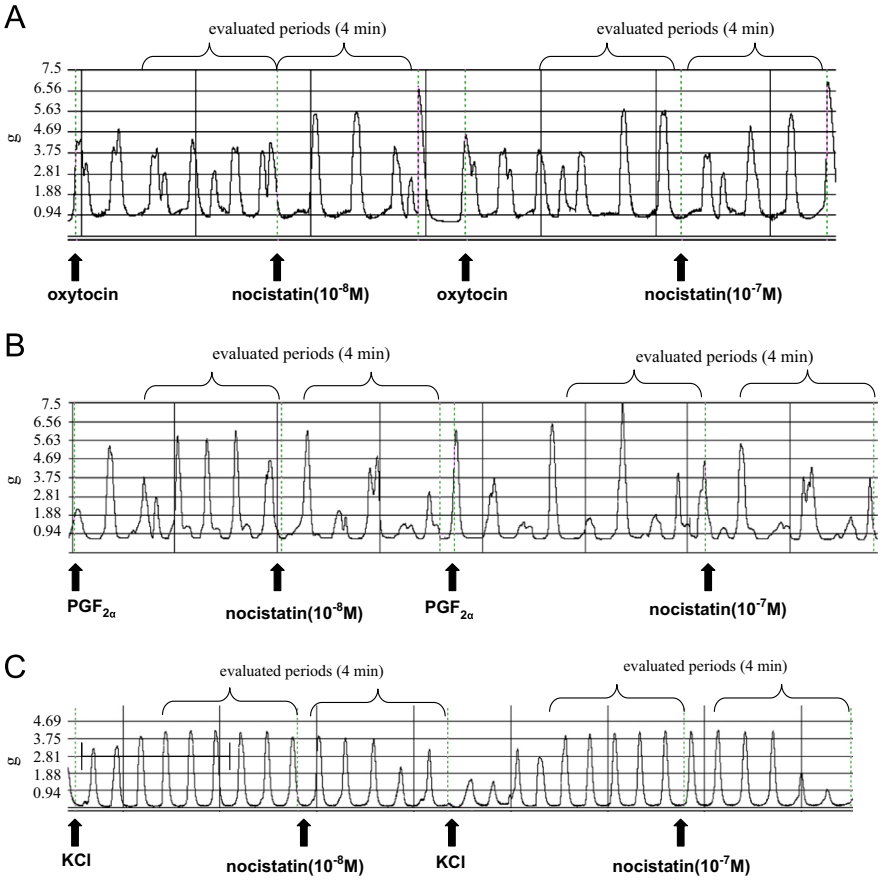

Fig. 2. Representative online recordings of the inhibitory effects of nocistatin on pregnant uterine contractions *in vitro*. The contractions were elicited with 10 nM oxytocin (A) or with 1 μM prostaglandin F2 alpha ($PGF_{2\alpha}$; B) or with 25 mM KCl (C). Nocistatin inhibited the prostaglandin F2 alpha and KCl-induced contractions, though it did not alter the oxytocin-evoked contractions.

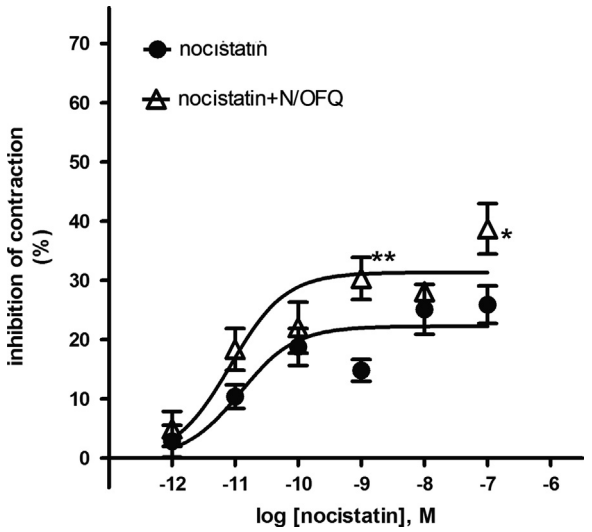

Fig. 3. Inhibitory effects of nocistatin and nociceptin (N/OFQ) on pregnant uterine contractions *in vitro*. The contractions were elicited with 1 μM prostaglandin F2 alpha. The concentration-dependent inhibitory effect of nocistatin (•) was increased by N/OFQ (△); $n=6$.

of the combination nocistatin+N/OFQ; $P<0.05$ (Fig. 4A). There was no significant difference between the log EC_{50} values (Table 2).

In the Ca^{2+}-poor environment, the concentration–response curves of nocistatin alone and of nocistatin in combination with naloxone were both shifted to the left as compared with the curves in standard de Jongh solution. The log EC_{50} values of nocistatin alone and of nocistatin with naloxone were significantly lower in the Ca^{2+}-poor environment than in standard de Jongh solution ($P<0.05$). At the same time, naloxone did not decrease the inhibitory effect of nocistatin in the Ca^{2+}-poor environment as it did in the standard buffer. The maximum contraction-inhibitory effect of nocistatin alone did not differ from that of nocistatin in combination with naloxone in the Ca^{2+}-poor buffer (Fig. 4B and Table 3).

3.3.3. *Investigation of the role of $K_{Ca}1.1$ channels in mediating the effects of nocistatin*

The effects of nocistatin on the spontaneous contractions of the term-pregnant rat uterus were also tested in the presence of the selective $K_{Ca}1.1$ channel inhibitor paxilline (5×10^{-6} M), (Fig. 5). In the presence of paxilline, the maximum contraction-inhibitory effect of nocistatin (E_{max}: $33.04\pm2.83\%$) was decreased significantly (E_{max}: $11.15\pm3.61\%$; $P<0.001$), while there was no significant difference between the log EC_{50} values (log EC_{50}: -8.29 ± 0.45 and -9.47 ± 0.93).

3.3.4. *Investigation of the role of CGRP in mediating the effects of nocistatin*

Since the exact site of action of nocistatin is still unclear, we tested whether it might act by modulating neuropeptide release from capsaicin-sensitive sensory nerve endings in the pregnant rat uterus. Neuropeptide depletion from the capsaicin-sensitive primary afferents was induced with capsaicin (Fig. 6). The maximum contraction-inhibitory effect of nocistatin was decreased significantly ($P<0.01$) after preincubation with capsaicin (1 μM; Table 4). The solvent of capsaicin (control) did not change the effect of nocistatin ($P>0.05$).

When the neuropeptide depletion was followed by the addition of CGRP (0.1 μM), the maximum contraction-inhibitory effect of nocistatin was significantly higher than after incubation with capsaicin ($P<0.01$). The addition of CGRP restored the inhibitory effect of nocistatin as compared with the control ($P>0.05$).

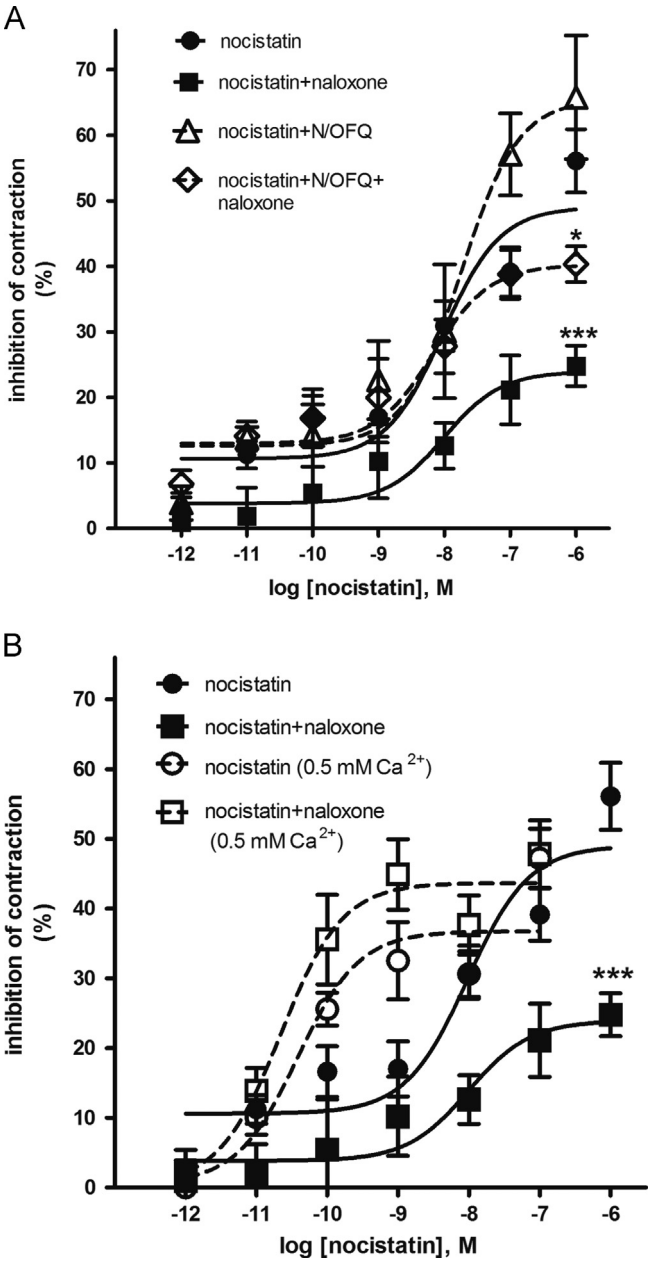

Fig. 4. Effects of nocistatin and naloxone on pregnant uterine contractions in a standard and a low Ca^{2+} environment *in vitro*. The contractions were elicited with 25 mM KCl in 22-day-pregnant rat uterine rings. The concentration-dependent inhibitory effect of nocistatin (•) was significantly attenuated by naloxone (■); $n=10$. When nocistatin, N/OFQ and naloxone were present, the joint effect of the three drugs (◊) was decreased as compared with the dual effect of nocistatin and N/OFQ (△); $n=6$ (A). The concentration-dependent inhibitory effect of nocistatin (○) was not altered by naloxone (□) in the Ca^{2+}-poor buffer, whereas it was inhibited in the standard Ca^{2+}-containing buffer (• nocistatin; ■ naloxone); $n=6$ (B).

3.4. *Measurement of uterine cAMP accumulation*

We also investigated whether cAMP accumulation plays a role in the contraction-inhibitory effect of nocistatin (Fig. 7). Nocistatin evoked a significant increase ($P<0.001$) in the uterine cAMP level (15.12 ± 0.40 pmol/mg tissue) as compared with the basic activity (12.82 ± 0.17 pmol/mg tissue). Co-administration of N/OFQ with nocistatin caused a further elevation in the cAMP level

Table 2
log EC_{50} and maximum contraction-inhibitory values of nocistatin alone and in the presence of nociceptin (N/OFQ) on prostaglandin $F2_{\alpha}$ ($PGF_{2\alpha}$) or KCl-stimulated contractions; and of nocistatin with N/OFQ+naloxone on KCl-stimulated contractions in the 22-day-pregnant rat uterus *in vitro* ($n=8$).

Substance	log EC_{50} ($\pm$ S.E.M.)		E_{max} (% $\pm$ S.E.M.)	
Nocistatin (on $PGF_{2\alpha}$-evoked contraction)	-10.92 ± 0.22		25.90 ± 3.16	
Nocistatin+N/OFQ	-11.07 ± 0.21	ns	38.71 ± 4.26	[a]
Nocistatin (on KCl-evoked contraction)	-8.02 ± 0.18		56.10 ± 4.82	
Nocistatin+N/OFQ	-7.75 ± 0.27	ns	65.78 ± 9.42	[a]
Nocistatin+N/OFQ+naloxone	-8.17 ± 0.27	[b]	40.33 ± 2.73	[c]

[a] $P<0.05$, ns: nonsignificant; significances are expressed relative to nocistatin alone.
[b] Nonsignificant; significances are expressed relative to nocistatin in the presence of N/OFQ.
[c] $P<0.05$.

Table 3
log EC_{50} and maximum contraction-inhibitory effects of nocistatin alone and in the presence of naloxone on KCl-stimulated uterine contractions in the 22-day-pregnant rat *in vitro*, either in standard Ca^{2+}-containing or in Ca^{2+}-poor de Jongh solutions ($n=6$).

Substance	log EC_{50} ($\pm$ S.E.M.)		E_{max} (% $\pm$ S.E.M.)	
Nocistatin (1 mM Ca^{2+})	-8.02 ± 0.18		56.10 ± 4.82	
Nocistatin+naloxone (1 mM Ca^{2+})	-8.01 ± 0.47	ns	24.78 ± 3.08	[a]
Nocistatin (0.5 mM Ca^{2+})	-10.42 ± 0.23	[b]	47.21 ± 4.27	
Nocistatin+naloxone (0.5 mM Ca^{2+})	-10.66 ± 0.24	ns; [b]	47.82 ± 4.85	ns

[a] $P<0.001$, ns: nonsignificant. The significances of the joint effect of nocistatin +naloxone are expressed relative to nocistatin alone in the same Ca^{2+}-containing de Jongh solution.
[b] $P<0.05$; significances are expressed relative to the same substance in a different Ca^{2+}-containing de Jongh solution.

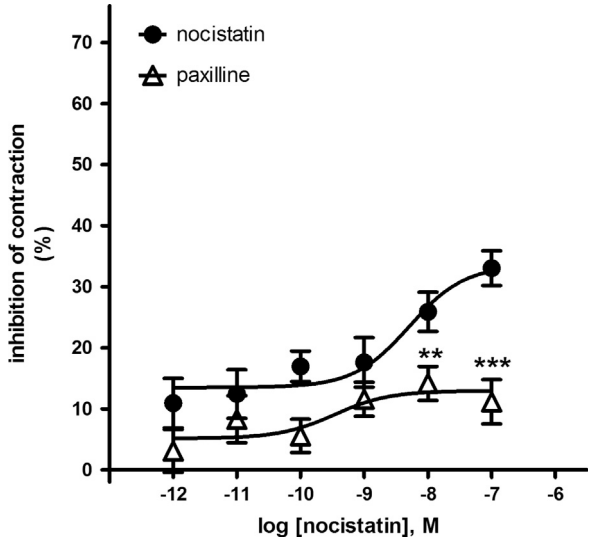

Fig. 5. Effects of paxilline on the contraction-inhibitory effect of nocistatin *in vitro*. The spontaneous contractions were recorded in 22-day-pregnant uterine rings from the rat. The concentration-dependent inhibitory effect of nocistatin (•) was decreased by paxilline (▲); $n=8$.

(17.48 ± 0.29 pmol/mg tissue; $P<0.01$). However, when naloxone was co-administered with nocistatin, a significant decrease was detected in the cAMP level (13.35 ± 0.52 pmol/mg tissue) as compared with the effect of nocistatin alone ($P<0.05$).

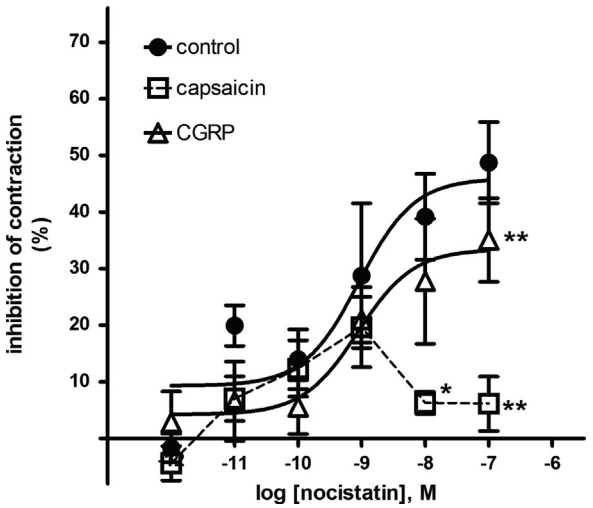

Fig. 6. The role of calcitonin gene-related peptide (CGRP) on the contraction-inhibitory effect of nocistatin *in vitro*. The contractions were elicited with 25 mM KCl in 22-day-pregnant rat uterine rings. The concentration-dependent inhibitory effect of nocistatin (•; control) was reduced significantly after preincubation with capsaicin (□; 1 μM). Addition of CGRP (0.1 μM) after preincubation with capsaicin caused a significant elevation of the concentration–response curve of nocistatin (△); $n=6$.

4. Discussion

Many studies have been reported on the central effects of nocistatin, but none on the peripheral effects of nocistatin. We earlier described the uterus-relaxant effect of N/OFQ, a peptide derived from the same precursor as nocistatin (Klukovits et al., 2010). Nocistatin displays opposite effects to those of N/OFQ in the central nervous system. Our present aims were to determine the peripheral effects of nocistatin on the rat uterus *in vitro*, and to elucidate its signalling mechanism.

PNOC mRNA is expressed predominantly in the central nervous system (the brain and spinal cord). A previous study also confirmed its presence in human peripheral blood mononuclear cells (Williams et al., 2008), and it has been detected in the rat ovary (Mollereau et al., 1996; Leo et al., 2001). However, the expressions of *PNOC* mRNA and nocistatin have not been investigated to date in the uterus. We found that the common precursor for N/OFQ and nocistatin, the *PNOC* mRNA, is expressed locally in the pregnant rat uterus, indicating that N/OFQ and nocistatin are synthesised locally. This finding justifies a local regulatory role for these pronociceptive peptides, in the term pregnant rat uterus.

In addition, we detected the presence of nocistatin in the pregnant and non-pregnant rat uterus. Both the expressions of *PNOC* mRNA and of nocistatin were high on the last day of pregnancy, as compared with earlier days. Furthermore, we found that nocistatin in the pregnant rat uterus at term is about 10 times more abundant than N/OFQ, so it seems that *PNOC* mRNA is translated mainly to nocistatin, rather than N/OFQ. The plasma level of nocistatin did not change at the end of pregnancy, which further confirms the local origin of nocistatin in the uterus.

After confirming that N/OFQ is effective in inhibiting uterine contractions (Klukovits et al., 2010), we investigated whether nocistatin influences the uterine smooth muscle *in vitro*. Although nocistatin possesses a contraction-inhibitory effect on KCl- and $PGF_{2\alpha}$-evoked contractions, its action against $PGF_{2\alpha}$ was weak. N/OFQ was able to potentiate the inhibitory effect of nocistatin in both cases. Nocistatin does not counteract the effects of N/OFQ on the myometrium contractility, as was presumed in previous studies relating to their actions in the central nervous system. However, nocistatin was ineffective on oxytocin-induced contractions. This phenomenon will

Table 4
log EC_{50} and maximum contraction-inhibitory effects of nocistatin after preincubation with capsaicin (1 μM), with the solvent of capsaicin (control) and with capsaicin (1 μM) and CGRP (0.1 μM) ($n=6$).

Substance	log EC_{50} (± S.E.M.)	Max. inhibition at 10^{-7} M nocistatin (% ± S.E.M.)	
Nocistatin control	−9.04 ± 0.43	48.72 ± 7.18	
Nocistatin preincubated with capsaicin	Not converged	6.14 ± 4.79	[a]
Nocistatin preincubated with capsaicin and CGRP	−9.06 ± 0.46	35.05 ± 7.38	[a]

[a] $P < 0.01$. Significances after preincubation with capsaicin are expressed relative to nocistatin alone, and significances after preincubation with capsaicin and CGRP &QJ; are expressed relative to preincubation with capsaicin alone.

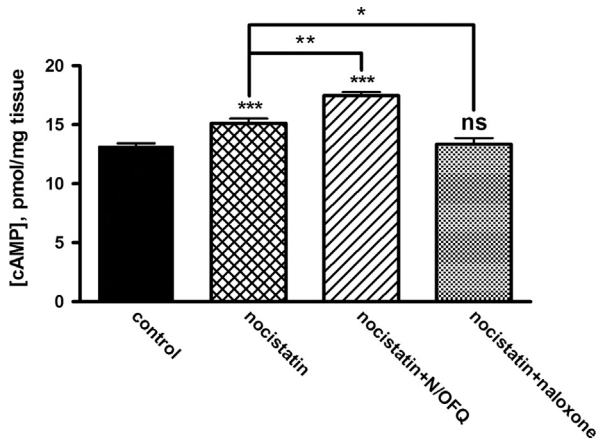

Fig. 7. Effects of nocistatin on intracellular cAMP levels in the pregnant rat uterus. Nocistatin caused a significant elevation in cAMP level as compared with the control (black column); ***$P < 0.001$. The presence of N/OFQ increased (**$P < 0.01$), while naloxone decreased (*$P < 0.05$) the nocistatin-induced intracellular cAMP accumulation.

be discussed later with regard to a detailed mechanism of the action of nocistatin on the pregnant rat myometrium.

Naloxone inhibits the contraction-inhibitory effect of nocistatin against KCl-induced contractions. Nocistatin does not bind to any of the classical opioid receptors, or to the NOP receptor (Johnson and Connor, 2007; Fantin et al., 2007), thus this inhibition is not likely to be mediated by opioid receptors. It was demonstrated that naloxone induces an increase in inward Ca^{2+} currents (Kai et al., 2002). These findings led us to investigate whether the inhibitory effect of naloxone on nocistatin-induced uterus relaxation is mediated by the opening of inward rectifying Ca^{2+} channels. To test our hypothesis, we used a Ca^{2+}-poor environment, where naloxone did not inhibit the effect of nocistatin. We presume that, in a low extracellular Ca^{2+} concentration, naloxone is probably unable to promote a Ca^{2+} influx, and hence it cannot overcome the relaxation induced by nocistatin. The leftward shift in the concentration–response curves are likely to be due to the weaker tissue contractility, caused by the lower Ca^{2+} content.

When N/OFQ was present, naloxone was able to decrease the common contraction-inhibitory effect of nocistatin and N/OFQ. However, we found earlier that naloxone increases the effect of N/OFQ (Klukovits et al., 2010). The effect seen here is probably the consequence of the more pronounced inhibitory effect of nocistatin as compared with that of N/OFQ.

After the description of the contraction-inhibitory effect of nocistatin in the *in vitro* contractility studies, our further aim was to investigate some of the potential nocistatin signalling pathways.

In the uterus, the plentiful $K_{Ca}1.1$ channels play an important role in decreasing depolarisation, thereby relaxing the uterine smooth muscle. These channels are activated by a cAMP-dependent phosphorylation pathway (Chanrachakul et al., 2004). We set out to investigate the role of K^+ currents in the intracellular signalling of nocistatin. Paxilline, a selective inhibitor of the $K_{Ca}1.1$ channels, inhibited the contraction-inhibitory effect of nocistatin, as evidence that the Ca^{2+}-dependent K^+ channels play a role in the intracellular signalling of nocistatin.

As regards the higher contractility during delivery, the elevation of nocistatin levels in the myometrium at term appears rather contradictory, since nocistatin has uterus relaxing effect. Nevertheless, it was also reported to inhibit hyperalgesia and allodynia, thus it has a regulatory role in pain signalling, which may explain its functional importance during labour. Additionally, inflammatory mediators play a role in the initiation of labour, yet some of them (*e.g.* CGRP) exhibit utero-relaxant activity among their various effects.

Opioid-like nociceptive peptides have been reported to release neurotransmitters such as CGRP or substance P (SP) from capsaicin-sensitive primary sensory neurons (Peiser et al., 2000; Helyes et al., 1997). These neuropeptides are synthesised in the dorsal root ganglia (DRG) cells, stored in vesicles and released by exocytosis (Lundberg, 1996) in response to electrical (Markowitz et al., 1987; Buzzi et al., 1991) or chemical stimulation (Kilo et al., 1997). CGRP has been reported to inhibit smooth muscle contractility in a variety of tissues, including the pregnant rat uterus (Pennefather et al., 1990). Other studies have reported that the binding of ^{125}I-CGRP to rat uterine membranes was increased during pregnancy and decreased during parturition (Yallampalli et al., 1999). It is known that denervation takes place in the myometrium during pregnancy, but this process mainly affects adrenergic nerves and does not involve the sensory units, including CGRP-containing nerves (Klukovits et al., 2004).

The potential involvement of CGRP in the actions of nocistatin on the pregnant rat uterus was therefore also tested. While capsaicin causes the depletion of CGRP from sensory nerve terminals (Holzer, 1991), other studies have furnished evidence of a CGRP reload into the sensory nerve terminals after depletion by capsaicin (Sams-Nielsen et al., 2001). We investigated the effect of nocistatin either on capsaicin-induced CGRP-depleted uterus samples or on CGRP-reloaded uterus samples. Capsaicin blocked the contraction-inhibitory effect of nocistatin, which was restored after the tissue samples were incubated with CGRP. Consequently, we assume that CGRP is an important factor in the contraction-inhibitory effect of nocistatin.

To support the hypothesis of the cross-talk between nocistatin and CGRP, a special population of opioid receptors in DRG neurons was reported, where low doses of opioids can provoke hyperalgesia, due to the activation of excitatory opioid receptors on the afferent nerve terminals (Crain and Shen, 2000). There is biochemical and pharmacological evidence that this phenomenon involves an increased release of excitatory neuropeptides, including CGRP and SP, in the spinal cord (Xu et al., 2003; Wiesenfeld-Hallin et al., 1991; Crain and Shen, 1990). We assume that nocistatin, similarly to opioid peptides and N/OFQ, may promote the release of neuropeptides from sensory nerves (Moran et al., 2000). This mechanism may explain the ineffectiveness of nocistatin against

oxytocin-induced contraction; it was reported that the CGRP-agonist adrenomedullin also failed to block oxytocin-evoked contractions, whereas it was effective against spontaneous and bradykinin-induced contractions (Yanagita et al., 2000).

For further investigation of the mechanism by which nocistatin inhibits uterine contractions, the effect of nocistatin on uterine cAMP accumulation was measured. We detected moderate elevation of cAMP levels in the presence of nocistatin, similarly as in the case of N/OFQ. In the presence of N/OFQ with nocistatin, a further cAMP level elevation was found, which can be explained by the mutual cAMP-accumulating effects of N/OFQ and nocistatin-induced CGRP liberation (Klukovits et al., 2010; Dong et al., 2005). In correlation with the *in vitro* contractility studies, naloxone decreased the cAMP levels elevated by nocistatin, which suggests that naloxone interferes with nocistatin at the level of G-protein activation, too.

Due to the CGRP-releasing effect of nocistatin, the relaxant mechanism seems to be an indirect pathway. On the other hand, the opening of potassium channels and inducing inward potassium current may also play a role in the regulation of the relaxing effect of nocistatin, and can be considered as direct effect on uterine smooth muscle. Thus nocistatin might have both direct and indirect dilatory effect on myometrium.

5. Conclusions

These results provide evidence that nocistatin alone and also in combination with N/OFQ, generated locally in the uterus, exert an inhibitory effect in the rat uterus, this mechanism being mediated mainly by $K_{Ca}1.1$ channels and consequent hyperpolarization, and by release of the sensory neuropeptide CGRP. Naloxone inhibits the contraction-inhibitory effect of nocistatin by activating inward rectifying Ca^{2+} channels and by decreasing the cAMP-accumulating effect of nocistatin. The elevated cAMP levels moderately contribute to the uterus-relaxing effect of nocistatin. This complex signalling pathway may provide an opportunity for the development of novel treatments for the inhibition of uterine contractions and hence prevent preterm birth. The findings of this *in vitro* study need to be evaluated under *in vivo* conditions, and further experiments on human tissue are necessary in order to allow conclusions on the relevance of the present findings as concerns human disease.

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